Smithsonian Tropical Research Institute

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PROYECTO MINA DE COBRE PANAMÁ

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CATEGORIA III

INFORME TRIMESTRAL DEL PROYECTO DE CONSERVACIÓN EX SITU DE ESPECIES DE INTÉRES DE ANFIBIOS

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INFORME TRIMESTRAL DEL PROYECTO

"LA CONSERVACIÓN EX SITU DE RANAS DEL ÁREA DE DONOSO, RENOVACIÓN POR TRES AÑOS"

Período: Febrero-Abril 2021

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Introducción

El 1 de septiembre de 2019, Minera Panamá S.A. (MPSA) y el Instituto Smithsonian de Investigaciones Tropicales (STRI) firmaron un acuerdo con el fin de extender el proyecto de conservación ex situ de anuros del área de Donoso por tres años. Con este acuerdo se continuarán los esfuerzos de conservación e investigación que contribuirán al manejo y preservación de los anfibios de Donoso, enfocándose en las especies de interés (EdI). Este proyecto es coordinado por el Proyecto de Rescate y Conservación de Anfibios de Panamá (PARC), que lleva a cabo las actividades de conservación ex situ dentro de instalaciones del STRI, ubicadas en Gamboa, provincia de Colón. En este informe se presentan los avances, de acuerdo a los objetivos acordados de este proyecto, realizados durante el período Febrero-Abril 2021.

Durante este trimestre, a pesar de las limitaciones resultado de la pandemia causada por el COVID-19, nosotros hemos continuamos brindándole todo el cuidado requerido a las poblaciones de anfibios que mantenemos en el programa de conservación ex situ en Gamboa; al mismo tiempo, evitando o minimizando el riesgo a la salud de nuestros colaboradores. Estas actividades se realizaron siguiendo las regulaciones y la autorización de las entidades de gobierno correspondientes y las medidas indicadas por el STRI. No obstante, varias actividades adicionales han estado suspendidas; las cuales, no están directamente vinculadas al cuidado y reproducción de los animales, como aquellas que están relacionadas con trabajo de campo. Estas actividades se tendrán que planificar y reprogramar en el futuro, cuando éstas se puedan realizar normalmente.

Objetivo 1. – Mantener la instalación para la cría y reproducción de poblaciones en cautividad de las especies de anfibios de interés de Donoso.

Debido a las restricciones por la pandemia del COVID-19, no ha sido posible seguir invirtiendo en la capacitación de personal; por lo que, no hemos recibido voluntarios, estudiantes, pasantes ni colaboradores de investigación en nuestras instalaciones durante estos meses. No obstante, el 21 de marzo, presentamos la charla titulada "Proyecto de Rescate y Conservación de Anfibios de Panamá: Conservación ex-situ de ranas de Donoso", los colaboradores de Cobre Panamá.

Realizamos trabajos de mantenimiento y reparación de equipo en la instalación de Gamboa, principalmente relacionados al reemplazo de aires acondicionados y lámparas, reparaciones de tuberías de agua, el mantenimiento del generador eléctrico y de la planta de tratamiento de

aguas residuales. Continuamos utilizando al último contenedor para la reproducción de ranas, el cual aún requiere ser habilitado completamente. A inicios de abril, finalizaron los trabajos de construcción de la pequeña edificación (150 m^2), por la empresa Kunkel Constructions Inc., destinada a la producción de invertebrados. Estamos por obtener el permiso para su ocupación; luego del cual, procederemos a instalar un sistema de rociadores contraincendios en esta edificación. En cuanto a los fondos para la financiación parcial de la construcción del edificio para la producción de invertebrados, STRI tomó la decisión de renunciar a estos fondos.

La producción de invertebrados continuó sin contratiempos e ininterrumpidamente en los salones temporales, ubicados en el área conocida como Santa Cruz. Continuamos produciendo grillos, dos especies de moscas de las frutas, colémbolos, larvas de polilla, lombrices de tierra, una especie de cucaracha, larvas de escarabajos ("mealworms"), escarabajos del frijol, cochinillas y tenebrios para alimentar a las poblaciones de anfibios en cautiverio. Además, iniciamos la producción de larvas de polillas de cera ("waxworms"), que son ricas en lípidos, para suplementar la dieta de los anfibios. Durante este trimestre, la producción de invertebrados sobrepasó el nivel requerido como alimento de las ranas que tenemos en Gamboa, lo cual es adecuado en caso de que ocurra una caída repentina en la producción de alimento para los anfibios.

Los números de individuos de las poblaciones en cautiverio de las especies de anfibios del área de Donoso, que manteníamos al 30 de abril de 2021, en Gamboa eran:

Especie	Machos	Hembras	Juveniles	Total	Reproducción en cautiverio	Renacuajos
Andinobates geminisae	40	79	52	171	+	75
Atelopus varius	312	223	180	715	+	~1300
Craugastor evanesco	22	25	5	52	-	-
Gastrotheca cornuta	7	1	0	8	-	-
Oophaga vicentei	16	26	5	47	+	~8
Total	**397**	**354**	**242**	**993**		**~1383**

Nota: *Craugastor evanesco* y *Gastrotheca cornuta* no presentan etapa de larvaria o renacuajo libre.

Al 30 de abril de 2021, teníamos 993 ranas de cinco especies de anuros del área de Donoso. Entre las especies, que se mantienen en cautiverio, están cinco Especies de Interés (EdI) del Proyecto Mina de Cobre Panamá: *Andinobates geminisae*, *Atelopus varius*, *Craugastor evanesco, Gastrotheca cornuta* y *Oophaga vicentei*.

Para las especies *Andinobates geminisae*, *Craugastor evanesco y Oophaga vicentei*, originalmente se colectaron suficientes individuos fundadores con el fin de iniciar la población ex situ. Sin embargo, es recomendable obtener más individuos de *Atelopus varius* (6-10 hembras de la población en Nuevo Petaquilla) y *Gastrotheca cornuta* (13 machos y 19 hembras) para obtener una representatividad adecuada de su diversidad genética. La especie *G. cornuta* ha sido particularmente difícil de encontrar; por lo que, nos estaremos concentrando en la búsqueda de más individuos de esta especie. Esperamos reanudar expediciones este año, con el fin de recolectar más fundadores de esta especie.

Durante este período, se mantuvieron 37 parejas de *Andinobates geminisae* en los tanques de reproducción y hubo varios eventos de reproducción de estas parejas. Resultado de los eventos reproductivos, para el final del período, se tenían 75 renacuajos y 52 juveniles. Adicionalmente, mantuvimos 21 parejas de *Oophaga vicentei* durante este período. Doce de estas parejas aún tienen puestas de huevos o renacuajos. Hemos observado ocho renacuajos de *O. vicentei*, algunos entre las plantas bromelias al final de este período. Debido a que no es recomendable manipular las bromelias para evitar la interrupción del cuidado parental de los huevos y renacuajos, no podemos establecer si hay más de éstos entre las plantas. A inicios de abril, añadimos pequeños recipientes con agua dentro de los tanques de reproducción de *O. vicentei* con el fin de incrementar la reproducción de estas ranas. Hemos observado que las hembras han empezado a utilizar estos recipientes para depositar sus renacuajos, al igual que las bromelias artificiales.

Pareja de *Craugastor evanesco* en tanque de reproducción, la hembra es más grande que el macho.

En este período, se hizo nuevamente una redistribución de parejas de ranas fundadoras (i.e., provenientes del campo) y de ranas nacidas en cautiverio, con el fin de estimular su reproducción. Como resultado, aumentamos el número de parejas de adultos de *Craugastor evanesco* que manteníamos en tanques para su reproducción, pasando de 16 parejas a mantener 26 parejas; sin embargo, éstas no se han reproducido aún. También, finalizó la salida de post-metamorfos de los tanques con renacuajos de las dos puestas de *Atelopus varius*, reportadas en el trimestre pasado. Adicionalmente, durante Febrero-Abril, se colocaron ocho parejas en tanques de reproducción. Solamente tres de estas parejas depositaron huevos, de los cuales mantenemos renacuajos. Al final del período, teníamos un total de aproximadamente 1300 renacuajos de las tres parejas, en tanques de reproducción.

Juveniles de *Atelopus varius* (linaje de tierras bajas) de unos tres meses de haberse transformado en ranas.

Como parte de los preparativos para un experimento en el laboratorio y un ensayo de liberación con *A. varius*, hemos realizado cruces de individuos cuyo linaje proviene de poblaciones de tierras bajas (i.e., Donoso) con individuos de linaje proveniente de poblaciones de tierras altas. Durante Febrero-Abril, finalizó la salida de post-metamorfos de los tanques con renacuajos de las puestas reportadas en el informe anterior. A finales de este período, mantenemos renacuajos de un nuevo cruce (i.e., ~800 renacuajos, no incluidos en el cuadro). Con este experimento y ensayo de liberación determinaremos si una mayor diversidad genética de los individuos confiere una mayor resistencia o tolerancia al hongo patógeno de anfibios, *Batrachochytrium dendrobatidis*.

Además, se mantuvo a dos parejas de *Gastrotheca cornuta* en tanques para su reproducción, cuyas hembras están grávidas; pero todavía no se han reproducido; a pesar de, que a los machos se les ha observado emitiendo llamados para atraer a las hembras. A través de estos intentos y eventos reproductivos, seguimos conformando paulatinamente poblaciones estables en cautiverio de las especies EdI de Donoso. En este sentido, para las especies que se han reproducido con mayor frecuencia, *Atelopus varius*, *Andinobates geminisae* y *Oophaga vicentei*, tenemos individuos fundadores (parentales) y descendientes de la primera generación filial (F1) y la segunda generación filial (F2) en las poblaciones de éstas en cautiverio. En el caso de *Craugastor evanesco* y *Gastrotheca cornuta*, solamente se tienen parentales y descendientes F1.

Pareja de *Gastrotheca cornuta* dentro del tanque para su reproducción. El macho al frente y la hembra en el fondo de la foto.

Durante este trimestre, se examinaron heces de las ranas para el control de endoparásitos en las poblaciones en cautiverio. Estamos completando el tratamiento antihelmíntico de todas las ranas que requerían desparasitarse. En cuanto al síndrome de patas débiles (SLS), no hubo post-metamorfos de *Andinobates geminisae*, *Atelopus varius* u *Oophaga vicentei* que presentaran este síndrome. La ausencia del SLS entre los post-metamorfos de estas especies puede considerarse como un logro en este período; ya que, los post-metamorfos con SLS no pueden moverse normalmente para alimentarse y, como consecuencia, mueren. La causa de este síndrome ha sido investigada por nosotros, tanto en especies del género *Atelopus*, como en *Andinobates geminisae*. Estas investigaciones han resultado en dos publicaciones en revistas científicas y, en este trimestre, en una tercera publicación (Anexo 1). Se trata de los resultados de un experimento sobre los efectos que tienen el grado de cuidado y la calidad del agua en la prevalencia del SLS en renacuajos de *A. geminisae*, del estudio liderizado por Kathleen Higgins, ex pasante de nuestro proyecto.

Además, se ha continuado con la introducción de datos de los individuos de Donoso en la base de datos “Zoological Information Management System” (ZIMS) (http://www2.isis.org), lo cual facilita el manejo del inventario de animales, el control de la conformación genética y demográfica de las colecciones de animales vivos, la identificación de animales no relacionados para realizar cruces, el mantenimiento de un registro del estado de salud y el tratamiento que se le ha dado a cada individuo, entre otros.

Objetivo 2. – Continuar el trabajo para ensayos de liberación de anfibios en el área de Donoso.

Para este trimestre, no teníamos programado realizar ensayos de liberación de anfibios, debido a que el trabajo de campo está suspendido hasta que se pueda retornar a la normalidad. Nuestro colaborador Dr. Luke Linhoff, becario postdoctoral del Instituto Smithsonian, no ha podido viajar a Panamá para realizar los ensayos *in vitro* que hacen falta y completar, así, las investigaciones sobre las secreciones de la piel (“mucosoma”) de las ranas. Como se indicó en el informe trimestral anterior, los resultados de estas investigaciones pueden tener implicaciones importantes para la liberación exitosa de ranas provenientes de poblaciones en cautiverio. Confiamos en que los trabajos relacionados a los ensayos de liberación se puedan reiniciar en los próximos meses.

Objetivo 3. – Realizar expediciones para recolectar ciertas especies de anfibios en riesgo en el área de Donoso.

Durante este período no realizamos expediciones para recolectar individuos. Como se indicó anteriormente, *Gastrotecha cornuta* es una Especie de Interés (EdI), de la cual se requieren más individuos fundadores para establecer adecuadamente una población ex situ en Gamboa. Esperamos realizar una expedición al sitio 4CS6, luego que se reestablezca la normalidad de las operaciones en el país.

Objetivo 4. - Estudio de los hongos patógenos de anfibios en la región de Donoso.

Debido a que no realizamos expediciones a Donoso en este trimestre, no obtuvimos muestras de piel para el análisis del hongo patógeno de anfibios, *Batrachochytrium dendrobatidis*.

Anexo 1. Artículo publicado: "Higgins, K., J. Guerrel, E. Lassiter, A. Mooers, W. J. Palen y R. Ibáñez. 2021. Observations on spindly leg syndrome in a captive population of *Andinobates geminisae*. Zoo Biology. https://onlinelibrary.wiley.com/doi/epdf/10.1002/zoo.21598."

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RESEARCH ARTICLE

ZOOBIOLOGY WILEY

Observations on spindly leg syndrome in a captive population of *Andinobates geminisae*

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Abstract

Amphibian health problems of unknown cause limit the success of the growing number of captive breeding programs. Spindly leg syndrome (SLS) is one such disease, where affected individuals with underdeveloped limbs often require euthanization. We experimentally evaluated husbandry-related factors of SLS in a captive population of the critically endangered frog, *Andinobates geminisae*. SLS has been linked to tadpole nutrition, vitamin B deficiency, water filtration methods, and water quality, but few of these have been experimentally tested. We tested the effects of water filtration method and vitamin supplementation (2017) and the effects of tadpole husbandry protocol intensity (2018) on time to metamorphosis and the occurrence of SLS. We found that vitamin supplementation and reconstituted reverse osmosis filtration of tadpole rearing water significantly reduced SLS prevalence and that reduced tadpole husbandry delayed time to metamorphosis. A fortuitous accident in 2018 resulted in a decrease in the phosphate content of rearing water, which afforded us an additional opportunity to assess the influence of phosphate on calcium sequestration. We found that tadpoles that had more time to sequester calcium for ossification during development had decreased the prevalence of SLS. Taken together, our results suggest that the qualities of the water used to rear tadpoles plays an important role in the development of SLS. Specifically, filtration method, vitamin supplementation, and calcium availability of tadpole rearing water may play important roles. Focused experiments are still needed, but our findings provide important information for amphibian captive rearing programs affected by high SLS prevalence.

KEYWORDS

amphibian calcium metabolism, amphibian captive husbandry, amphibian health, *Andinobates geminisae*, Anura, bone development, captive breeding, Dendrobatidae, metamorphosis, spindly leg syndrome (SLS), tadpole

1 | INTRODUCTION

Nearly 40% of all amphibian species are threatened with extinction (Gratwicke et al., 2012; Hoffmann et al., 2010; Scheele et al., 2019; Stuart et al., 2004). Conservation captive breeding is one important amphibian conservation action (Claunch & Augustine, 2015; Harding et al., 2016; Mendelson et al., 2006; Stuart et al., 2004). Though supposedly tolerant to the conditions of captivity (Balmford et al., 1996; Bloxam & Tonge, 1995; Conde et al., 2015), only 52% of amphibian captive breeding programs are successful (Biega, 2017).

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This low success rate is at least partially due to health problems resulting from inadequate knowledge of environmental and nutritional needs (Ferrie et al., 2014).

Spindly leg syndrome (SLS) is a disease characterized by the deformation and/or underdevelopment of the limbs during metamorphosis (Camperio Ciani et al., 2018; Claunch & Augustine, 2015; Hakvoort et al., 1995; Marlett et al., 1988). If not euthanized, metamorphs with SLS usually drown or die of starvation because of their restricted mobility (Claunch & Augustine, 2015; Marlett et al., 1988; Wright & Whitaker, 2001). SLS has been reported in a wide variety of anuran taxa with free-swimming tadpoles, for example, Dendrobatidae (poison dart frogs), *Bombina*, *Phyllomedusa*, *Discoglossus* (Hakvoort et al., 1995), *Mantella* (Claunch & Augustine, 2015), *Atelopus*, *Hyloscirtus* (Camperio Ciani et al., 2018). Hundred percent of captive born offspring may die in severe cases (Claunch & Augustine, 2015; Marlett et al., 1988). While there are many putative causes of SLS circulating in the zoo, veterinary, and hobbyist circles, experimental tests are scarce (Table 1).

The only published preventative treatment for SLS recommended in veterinary literature is the addition of vitamin B to tadpole rearing water (VEDCO®, containing vitamins B_1 through B_3, B_5, B_6, and B_{12}), which according to Wright and Whitaker (2001) reduced the prevalence of SLS in tadpoles of *Epipedobates* spp., *Dendrobates* ssp., and *Phyllobates* spp. when added to the water at a dosage of 0.5–1 ml/gallon. A related popular belief is that SLS is caused by folic acid (vitamin B_9) deficiency, based on the fact that folic acid deficiency in pregnant humans can lead to congenital limb deformities (Cleves et al., 2011). More recently, Camperio Ciani et al. (2018) found that the filtration method of tadpole rearing water was an important predictor of SLS prevalence in *Atelopus glyphus*. Tadpole feeding frequency and amount can have direct impacts on water quality, which is also suspected to play an important role on tadpole health, development, and SLS occurrence (Álvarez & Nicieza, 2002; Browne et al., 2003; Marlett et al., 1988; Wright & Whitaker, 2001).

We evaluated the role of environmental factors on SLS prevalence using the critically endangered frog, *Andinobates geminisae*, a focal species of ex-situ conservation efforts at the Panama Amphibian Research and Conservation Project (PARCP) in Gamboa, Panama. In an attempt to replicate the observations of Wright and Whitaker (2001) and assess the validity of the purported folic acid—SLS link, we examined the prevalence of SLS in metamorphs in response to the addition of VEDCO® vitamin B supplement and folic acid to the tadpole rearing water during a pilot experiment in 2017 (Experiment 1). We also tested the use of reconstituted reverse osmosis water for rearing tadpoles compared with carbon-filtered water to determine whether Camperio Ciani et al.'s (2018) findings were replicable in *A. geminisae*.

TABLE 1 Summary of experimentally tested husbandry-related causes of SLS

Experimental variable	Model Species (tadpoles)	Result	Source
Different types of commercial diets (dried brine shrimp, rabbit chow, baby food, fish food, dried tubifex worms, *Xenopus* tadpole diet)	*Dendrobates auratus*	There was no effect of commercial diet type on SLS.	Marlett et al. (1988)
Dietary calcium	*Lithobates catesbeianus*	Lower dietary calcium significantly increased skeletal deformities, including "crooked limbs," for tadpoles ≤ stage 18.	Marshall et al. (1980)
Environmental calcium	*Epipedobates tricolor*	Inconclusive: tadpoles died or developed poorly at all calcium concentrations.	Hakvoort & Gouda (1990)
Overfeeding	*Atelopus glyphus*	Overfeeding significantly increased SLS prevalence, especially if tadpoles were raised in carbon-filtered water instead of reconstituted reverse osmosis water.	Camperio Ciani et al. (2018)
Experimental versus commercial diet	*Atelopus glyphus*	There was no difference between experimental and commercial diet on SLS.	Camperio Ciani et al. (2018)
Water filtration method (reconstituted reverse osmosis water vs. carbon-filtered water)	*Atelopus glyphus*	Raising tadpoles in reconstituted reverse osmosis water significantly reduced SLS prevalence.	Camperio Ciani et al. (2018)
Diet ration	*Atelopus glyphus*	Ration had a significant positive effect on SLS in carbon-filtered water and increased the effect of filtration method on SLS.	Camperio Ciani et al. (2018)
Dietary protein	*Atelopus certus*	There was no effect of dietary protein on SLS.	Camperio Ciani et al. (2018)
Time into the breeding season	*Dendrobates auratus*	SLS prevalence increased with increased time into the breeding season.	Marlett et al. (1988)

Abbreviation: SLS, spindly leg syndrome.

Recorded prevalence of SLS in *A. geminisae* metamorphs changed from 58% (2016, unpublished data) to 3% (2017, data reported herein) after starting Experiment 1. Two possible SLS factors were knowingly changed before the Experiment 1: tadpole feeding and water change frequency were increased and the diet of the breeding adults was changed from cricket nymphs twice a week plus springtails once a week, to cricket nymphs three times a week. We ran a second experiment to test the hypothesis that tadpole husbandry intensity (feeding frequency and amount, and water change frequency) is a key SLS factor. We tested for an association between tadpole water quality and SLS prevalence by manipulating tadpole feeding and cleaning protocols in two treatment groups and tracked SLS prevalence and water quality in each group. We also measured tadpole survival and time to metamorphosis, with a view to informing efficient allocation of staff and time resources to amphibian husbandry. We predicted that tadpoles raised under intensive feeding and cleaning protocols would have a significantly lower SLS prevalence those raised under low intensity husbandry protocols.

2 | MATERIALS AND METHODS

The protocols employed in this study were reviewed and approved by the Canadian Council on Animal Care and the Smithsonian Tropical Research Institute Animal Care and Use Committee.

3 | STUDY SPECIES AND HUSBANDRY

A. geminisae is a critically endangered species of dendrobatid frog endemic to a small area in the Donoso region, Colón Province, Panama currently adjacent to a copper mining concession area (Batista et al., 2014; IUCN SSC Amphibian Specialist Group, 2019). The Panama Amphibian Research and Conservation Project (PARCP) initiated captive assurance populations of *A. geminisae* in 2013 in response to concerns about the impacts of chytridiomycosis and the potential loss of habitat.

We used tadpoles from six different breeding pairs, where each breeding pair and their tadpoles were housed in a separate 20 gallon breeding tank, fitted with a standardized set of oviposition/tadpole deposition sites (Figure 1), maintained at 24–25°C, 12:12 h dark:-light, 89%–100% relative humidity, misted with carbon-filtered water for 5 min every 3 h, and cleaned every 3–4 weeks. Adults were fed a standardized diet of cricket nymphs gutloaded with Mazuri® Hi Calcium Gut Loading Diet.

Between July 6 and October 2, 2017 (Experiment 1) and March 4 and May 16, 2018 (Experiment 2), *A. geminisae* females laid clutches of one to three eggs in capped PVC pipes (Figure 1) and the males transported hatched tadpoles to one of several water-filled petri dishes or bromeliad axils for development. We surveyed the breeding tanks three times per week to find clutches (on the male's back or in a deposition pool) and to track their development. This allowed us to collect all tadpoles from their deposition pool within 3 days of hatching. We used a new disposable pipette to move each tadpole from their original pool to an individual randomly assigned enclosed petri dish (filled with 12 ml of water) for development (Figure 1). In Experiment 1, tadpoles were fed 0.005 ± 0.002 g crushed Tetramin® fish flakes and given 50% water changes three times weekly. In Experiment 2, feeding and water change protocol varied by treatment.

FIGURE 1 Habitat design of parental breeding tanks with tadpoles in the front area. Each breeding tank had an identical set of objects: 10 capped pieces of polyvinyl chloride (PVC) pipe, 6 water-filled petri dishes, a pot hide, a small bromeliad, a clear shelter, and 22 numbered petri dishes

4 | EXPERIMENT 1

We raised *A. geminisae* tadpoles ($n = 97$) in one of four different water treatments groups: (1) unsupplemented reconstituted reverse osmosis water ($n = 25$), (2) unsupplemented carbon-filtered water ($n = 24$), (3) carbon-filtered water with Vedco® vitamin B complex added at a concentration of 300 ppm ($n = 24$), or (4) carbon-filtered water with folic acid added at a concentration of 417 ppm ($n = 24$). We used a partial factorial design due to the sample size limit of the number of offspring produced from six breeding pairs over 4 months. The concentrations of the vitamin B complex and folic acid added to the water were calculated using Wright and Whitaker's (2001) recommended dosage of 1 ml per gallon vitamin B complex. Water in treatment groups two to four was filtered with a mechanical and carbon filtration unit and passed through a phosphate reactor unit to remove excess phosphates before use. We compared the survival with metamorphosis, metamorphosis time (measured as the number

of days from birth to the emergence of both forelimbs ± 2 days), and SLS prevalence in metamorphs across treatments.

Tadpoles were assigned sequentially to treatments within each tank randomly without replacement to decrease the likelihood that tadpoles from the same clutch would be placed into the same treatment group. We replaced the initial carbon-filtered water with treatment-specific water via 50% water changes three times weekly. The position of each tadpole's dish in a tank was randomized three times per week to control for positional effects.

4.1 | Statistics

Two experimental variables (water filtration method and vitamin supplementation) were represented in the four treatment groups. We subset our data into treatment groups 2–4 to address the effect of vitamin supplementation and treatment groups 1–2 to address the effects of water filtration method on our response variables. To control for any effects of parental identity and tank environment, we included tank ID as a random effect in generalized linear mixed effect models of metamorphosis time, mortality, and SLS in response to each water treatment variable. We used likelihood ratio tests (LRTs) of null (response variable ~ 1 + (1|tank. ID)) and alternate (response variable ~ predictor + (1|tank. ID)) models to estimate predictor *p* values, considering a predictor to be "significant" if its addition to a model improved the fit such that the LRT *p* value was < .05.

5 | EXPERIMENT 2

Between March 4 and August 16, 2018, we raised *A. geminisae* tadpoles ($n = 60$) under one of two husbandry treatments: the intensive husbandry treatment and the medium husbandry treatment (Table 2). Our response variables were tadpole survival, time to metamorphosis, and SLS prevalence. We assigned the first tadpole collected from each breeding tank to one of two husbandry treatments (intensive or medium) randomly, and treatments were alternated for each subsequent tadpole. The position of each tadpole's dish in the front of the tank was randomized once a week to control for any positional effects. Only carbon-filtered water was used, changed three times per week (intensive husbandry protocol) or once every 2 weeks (medium husbandry protocol) (Table 2), with 6 ml of water removed and immediately returned three times per week in the medium husbandry treatment to control for disturbance effects. We checked the status of each tadpole during water changes. Tadpoles in the intense husbandry protocol group were fed 0.003 ± 0.002 g crushed Tetramin® fish flakes three times a week, and tadpoles in the medium husbandry protocol group were fed 0.005 ± 0.002 g crushed Tetramin® fish flakes once a week (Table 2).

5.1 | Water sampling protocol and water chemistry analysis

Every 2 weeks we retained 6 ml of waste water from the first two to three tadpoles added to each treatment group within each tank to test for ammonia (ppm NH_3-N using a Hanna® Instruments H1700), phosphates ppm PO_4^{3-} using Quantofix® 91320), pH (using a Macherey-Nagel 92110), total dissolved solids (ppm TDS, using Hanna® Instruments GroLine HI98131), conductivity (μS/cm using a Hanna® Instruments GroLine HI98131), nitrites/nitrates (ppm NO_2-N/NO_3-N using a Hach® 2745425), and total hardness (net dissolved calcium and magnesium hardness, represented as ppm $CaCO^{3-}$ using a Hach® 1194T51EA). We ran a multiparameter analysis of the source water (using a Hach® DR/890, YSI Professional Plus multiparameter instrument; see Figure 2) on April 26, March 5, May 24, and July 26, 2018 to evaluate the chemistry of the water used in the second experiment. Halfway through the experiment, we discovered that the phosphate reactor used to remove excess phosphates from the tadpole rearing source water was malfunctioning. Our water quality data were not designed to capture the effects of a broken phosphate reactor, so in July 2018 we measured the phosphate and calcium of the source water before and after passing through the repaired phosphate reactor to estimate the impact the broken phosphate reactor would have had on these two key parameters, with the assumption that the effects of the phosphate reactor did not change over time.

5.2 | Statistics

The malfunction of the phosphate reactor introduced the variable of phosphate concentration, but we could not include phosphate concentration in our models because only a subset of tadpole wastewater was sampled. Instead, we created a calcium (Ca^{2+}) sequestering time variable. Tadpoles are known to store calcium in specialized sacs during the growth period after hatching until just before metamorphic climax (Pilkington & Simkiss, 1966), and

TABLE 2 Summary of the differences in tadpole care protocol between the two husbandry intensity treatment groups

Care parameter	Husbandry Treatment	
	Intensive	Medium
Feeding dose	0.003 ± 0.002 g crushed fish flakes per feeding.	0.005 ± 0.002 g crushed fish flakes per feeding.
Feeding frequency	Tadpoles fed three times weekly.	Tadpoles fed once weekly.
Water change frequency	50% water change three times weekly.	50% water change once every 14 days.

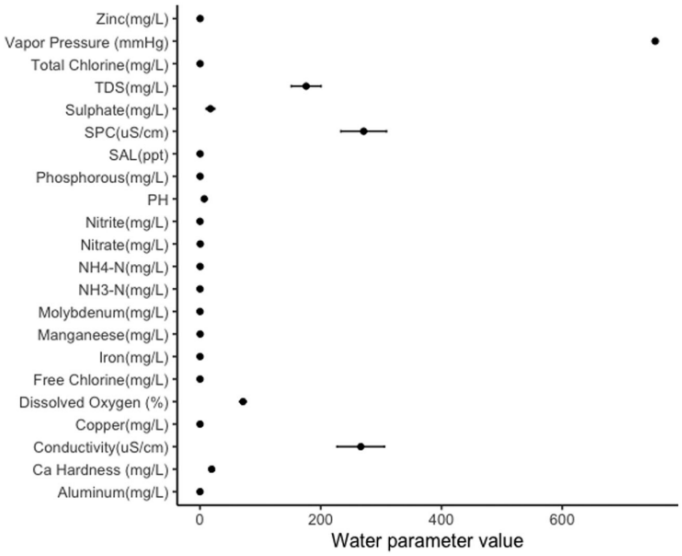

FIGURE 2 Mean values of various water parameters covered during full spectrum analyses of PARCP *A. geminisae* source water during Experiment 2. The mean for each parameter is taken from $n = 3$ or $n = 4$ measurements taken over the 6-month period of the experiment. Whiskers represent the standard error of the mean. PARCP, Panama Amphibian Research and Conservation Project

phosphate sequesters dissolved calcium in aqueous solution to form insoluble calcium phosphate salt (National Center for Biotechnology Information, 2019). Ca^{2+} sequestering time was used as a proxy for the amount of time each tadpole had to sequester calcium while unaffected by potentially high phosphates in the water, measured as the time difference (in days) between the repair of the phosphate reactor and metamorphic climax. We included Ca^{2+} sequestering time as a fixed effect in our models of SLS in response to husbandry treatment, with a random effect of tank ID.

Ca^{2+} sequestering time could not be used in models of survival to metamorphosis or metamorphosis time because its calculation depends on the date of emergence of forelimbs. To account for the effects of phosphate exposure on tadpole survival and time to metamorphosis, we considered a closely related variable, % PO_4^{3-} exposure, measured as the percent of the total lifetime of each tadpole that was spent in potentially high phosphates:

$$\%PO_4^{3-}\text{exposure} = \frac{\%PO_4^{3-}\text{reactor repair date} - \text{tadpole birth date}}{\text{date of metamorphosis} - \text{tadpole birth date}} \times 100.$$

Ca^{2+} sequestering time and % PO_4^{3-} exposure are strongly correlated ($r = -.86$, $n = 45$). % PO_4^{3-} exposure was treated as a fixed effect in models of survival and metamorphosis time in response to husbandry treatment, with a random effect of tank ID.

We used generalized linear mixed effects models (GLM or GLMERs; lme4 package (Bates et al., 2015) in R (R Core Team), to analyze the effects of husbandry treatment, Ca^{2+} sequestering time, and their interaction, on SLS, and husbandry treatment, % PO_4^{3-} exposure, and their interaction, on metamorphosis time and survival to metamorphosis. We controlled for possible effects of parental identity and tank environment by including tank ID as a random effect in all of our models. We used LRT of nested models to estimate predictor p values, considering a predictor "significant" if its addition to a model improved the fit such that the LRT p value was <.05. To see whether there was a link between the metamorphosis time of tadpoles and the development of SLS, we compared the mean metamorphosis time of tadpoles that developed SLS to tadpoles that did not using the Kruskal–Wallis test (Kruskal & Wallis, 1952).

To assess the effects of husbandry treatment on the water chemistry of individual tadpoles, we modeled each parameter (ammonia, pH, phosphates, total dissolved solids, conductivity, nitrites/nitrates, and total hardness) in response to husbandry treatment with tadpole age included as a random effect. For each water parameter, we used LRTs of null (parameter ~ 1 + (1| tadpole age)) and alternate (parameter ~ husbandry treatment + (1| tadpole age)) models to estimate predictor p-values. We considered a predictor "significant" if its addition to the model improved the fit such that the LRT p value was <.05.

We used the Kruskal–Wallis test (Kruskal & Wallis, 1952) to compare the mean calcium and phosphate values before and after phosphate removal in July 2018. These values were informally compared with the phosphate and calcium values taken from point measurments.

6 | RESULTS

6.1 | Experiment 1

A total of 97 tadpoles were born during Experiment 1, and 10 died before metamorphosis. There was no effect of vitamin supplementation or filtration method on survival or metamorphosis time

TABLE 3 Likelihood ratio test results on (a) the effect of vitamin supplementation of tadpole rearing water and (b) the effect of tadpole rearing water filtration method, on tadpole survival, time to metamorphosis, and SLS

	χ^2	*df*	*p*
	A. Vitamin B supplementation (treatment groups 2–4)		
Survival (*n* = 72)	0.7749	2	.6788
Metamorphosis time (*n* = 63)	2.9121	2	.2332
SLS (*n* = 63)	10.159	2	**.006229**
	B. Filtration method (treatment groups 1–2)		
Mortality (*n* = 49)	1.2262	1	.2681
Metamorphosis time (*n* = 45)	1.0377	1	.3084
SLS (*n* = 45)	4.2801	1	**.03856**

Note: Data from four treatment groups were subset into two groups to address the effects of each experimental variable separately. Bold-face values indicate statistical significance.

Abbreviation: SLS, spindly leg syndrome.

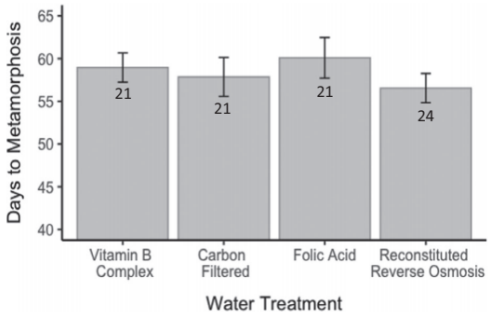

FIGURE 3 Mean and standard error of metamorphosis time for tadpoles raised in one of four water filtration and vitamin supplementation treatments in Experiment 1. Numbers at the top of each bar represent the number of observations in each treatment group. *N* = 87 tadpoles

(Table 3), which was on average 58 days across all treatments (*n* = 87, *SD* = 4.6, Figures 3 and 4a). Vitamin supplementation was a significant predictor of SLS (Table 3); None of the 42 tadpoles raised in carbon-filtered water supplemented with vitamin B complex or folic acid exhibited SLS, compared with four SLS metamorphs from 21 tadpoles raised in un-supplemented carbon-filtered water (Figure 4b). Raising tadpoles in reconstituted reverse osmosis water had a significant negative effect on SLS (Table 3), with one out of 24 tadpoles raised in reconstituted reverse osmosis water developing SLS, compared with 4 out of 21 tadpoles raised in carbon-filtered water (Figure 4b).

6.2 | Experiment 2

Tadpoles in the medium husbandry treatment took ~30 days longer to metamorphose ($\bar{x}$ = 93, *SD* = 15, *n* = 21) than tadpoles in the intensive husbandry treatment ($\bar{x}$ = 60, *SD* = 4, *n* = 24; Figure 5). This effect of husbandry treatment on metamorphosis time was significant. The addition of husbandry treatment to a null model of metamorphosis time improved the fit of the model significantly (LRT *p* value = 1.411e−15, χ^2 = 63.752, *df* = 1) as did the addition of husbandry treatment to a model of metamorphosis time in response to phosphate exposure (*p* = 1.582e−14, χ^2 = 58.994, *df* = 1). Phosphate exposure had a marginal effect on metamorphosis time (addition of phosphate exposure to a model of metamorphosis time in response to husbandry treatment gave LRT *p* = .05183, χ^2 = 3.7812, *df* = 1). We found no effect of phosphate exposure: husbandry treatment interaction on metamorphosis time (the inclusion of interaction term in models of metamorphosis time gave LRT *p* = .3031, χ^2 = 1.0607, *df* = 1). There was no difference in the metamorphosis time of tadpoles that developed SLS compared with tadpoles that did not (*p* = .6236, Kruskal–Wallis χ^2 = 0.24079, *df* = 1).

Husbandry treatment had no effect on the prevalence of SLS (addition of husbandry treatment to a null model of SLS gave LRT *p* = .3249, χ^2 = 0.969, *df* = 1; addition of husbandry treatment to a model of SLS in response to calcium sequestering time gave LRT *p* = 1, χ^2 = 0, *df* = 1). Calcium sequestering time had a significant negative effect on SLS (addition of calcium sequestering time to null model of SLS LRT *p* = 7.745e−05, χ^2 = 15.619, *df* = 1; addition of calcium sequestering time to a model of SLS in response to husbandry treatment LRT *p* = .000183, χ^2 = 13.998, *df* = 1; Figure 6), and there was a significant effect of husbandry treatment-calcium sequestering time interaction on SLS whereby the effect of calcium sequestering time on SLS was largest in the medium husbandry treatment group (inclusion of interaction term in addition to calcium sequestering time and husbandry treatment models gave LRT *p* = .01445, χ^2 = 5.9829, *df* = 1; Figure 6).

Six of 30 tadpoles in the intensive husbandry treatment died before metamorphosis and 9 of 30 tadpoles died in the medium husbandry treatment, but the effect of husbandry treatment on tadpole survival was not significant (addition of husbandry treatment to null model of survival LRT *p* = .3945, χ^2 = 0.7252, *df* = 1 and addition of husbandry treatment to model of survival in response to phosphate exposure LRT *p* = .1556, χ^2 = 2.0167, *df* = 1). Phosphate exposure had a marginal effect on tadpole survival; the addition of phosphate exposure to a null model of survival had an insignificant effect on model fit (LRT *p* = .08846, χ^2 = 2.9022, *df* = 1), but the addition of phosphate exposure to a model of survival in response to husbandry treatment had a significant effect on model fit (LRT *p* = .04, χ^2 = 4.1938, *df* = 1). There was no significant phosphate exposure: husbandry treatment interaction effect on survival (inclusion of interaction term LRT *p* = .2494, χ^2 = 1.3268, *df* = 1).

Wastewater from tadpoles reared under the medium husbandry treatment had significantly higher phosphates (LRT *p* = 4.527e−06, χ^2 = 21.028, *df* = 1), nitrates (*p* = .005102, χ^2 = 7.843, *df* = 1), nitrites

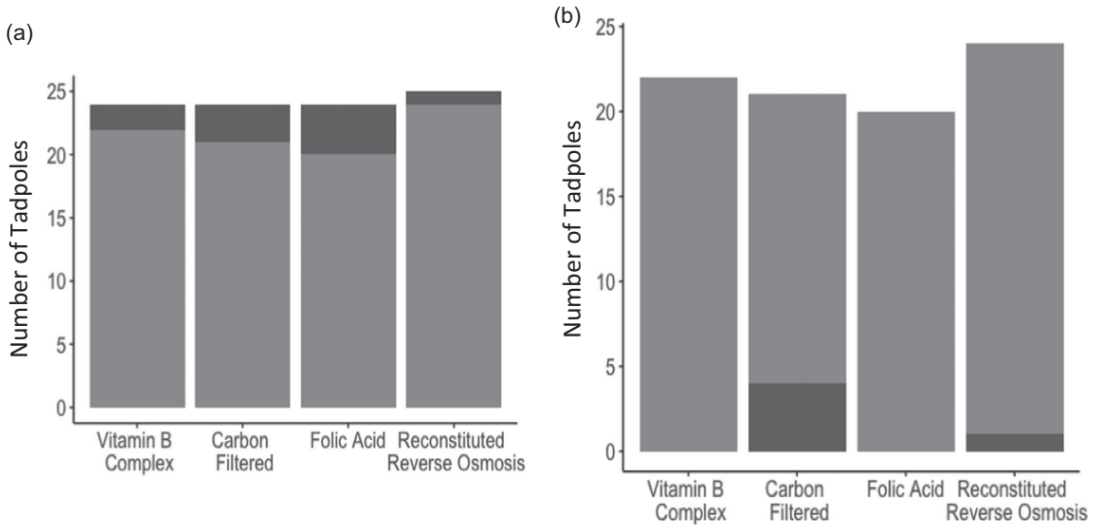

FIGURE 4 (a) The number of tadpoles that died before metamorphosis (dark gray) or survived to metamorphosis (light gray) in each treatment group in Experiment 1. $N = 97$ tadpoles. (b) The number of tadpoles to metamorphose with SLS (dark gray) or healthy (light gray) from each water treatment group in Experiment 1. $N = 87$ tadpoles

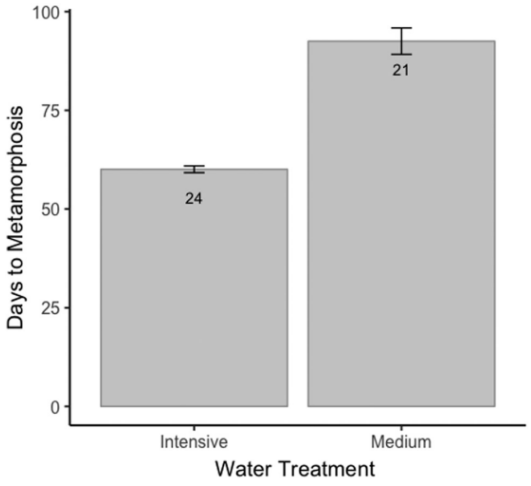

FIGURE 5 Mean ± standard error of the metamorphosis time of tadpoles raised under medium and high intensity husbandry treatments in Experiment 2. Numbers at the top of the bars represent the number of observations in each treatment group. $N = 45$ tadpoles

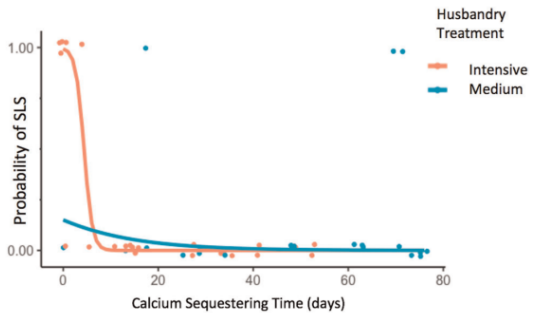

FIGURE 6 The probability of SLS (represented by the fitted line) over increasing calcium-sequestering time, generated from our model of SLS in response to increasing calcium exposure ($AIC\omega = 1$), with husbandry treatment and breeding tank included as a random effect for Experiment 2. The circles represent the SLS status (1 = SLS, 0 = healthy) and calcium sequestering time of each experimental tadpoles ($N = 45$). SLS, spindly leg syndrome

($p = .005102$, $\chi^2 = 7.843$, $df = 1$), total dissolved solids ($p = 7.138e{-}13$, $\chi^2 = 51.506$, $df = 1$) and conductivity ($p = 1.463e{-}13$, $\chi^2 = 54.619$, $df = 1$) than from tadpoles in the intensive husbandry treatment.

The level of phosphates in tested tadpole rearing water decreased from ~0.4 ppm PO_4^{3-} (before the phosphate reactor was known to be broken) to 0–0.04 ppm PO_4^{3-} after the phosphate reactor was repaired (Figure 7). Dissolved calcium levels varied between 15.6 and 20.8 ppm $CaCO_3$ over the course of the experiment (Figure 7). Water that had been treated by the phosphate reactor had a significantly lower concentration of phosphates than the source water ($p = .001434$, Kruskal–Wallis $\chi^2 = 10.161$, $df = 1$) while the calcium concentration remained constant (Figure 8). Phosphate measured from individual tadpole wastewater fluctuated between 0 and 100 mg/L over the course of the experiment, while calcium hardness stayed at 120 mg/L (Figure 7).

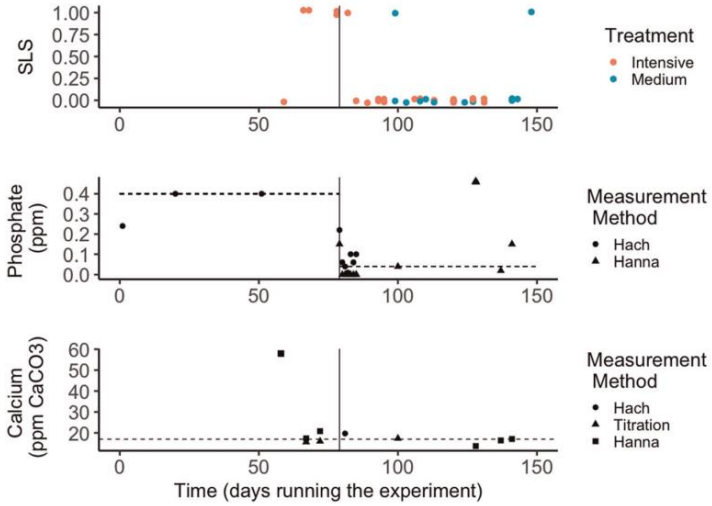

FIGURE 7 Comparison of SLS at metamorphosis, PO_4^{3-} concentration, and calcium concentration of the source water used for tadpoles, over the duration of Experiment 2. The vertical line at Day 79 represents the day that the phosphate reactor was repaired. In the top graph, each (jittered) point represents the metamorphosis of a single tadpole, with 1 indicating that the emerging metamorph had SLS and 0 indicating they were healthy. We used three methods to measure PO_4^{3-} and calcium: a "Hanna" colorimeter, a "Hach" colorimeter, and manual titration. The dashed lines represent the median phosphate (middle panel) or calcium (bottom panel) concentration of the water before and after the phosphate reactor was repaired (indicated by the vertical line)

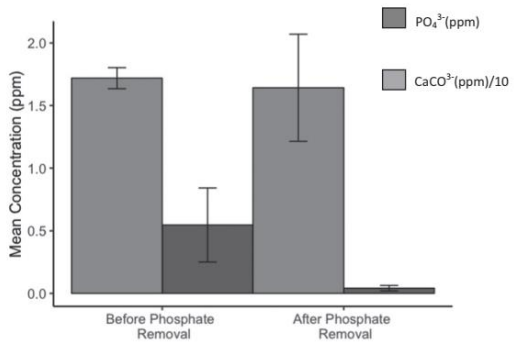

FIGURE 8 Mean phosphate (PO_4^{3-}) and calcium concentration ($CaCO_3$) of the tadpole source water before and after passing through the repaired phosphate reactor in July 2018. Error bars represent 95% confidence intervals. Concentrations are in mg/L, and calcium concentration is divided by a factor of ten for visualization on the same scale as phosphate data

7 | DISCUSSION

7.1 | Experiment 1

We found that raising tadpoles in reconstituted reverse osmosis water decreased the occurrence of SLS, consistent with the results of Camperio Ciani et al. (2018), who found that SLS was reduced in *Atelopus glyphus* tadpoles that were raised in reconstituted reverse osmosis water compared with carbon-filtered water. While the specific elements involved in this effect are not known, Camperio Ciani et al. (2018) postulated that calcium concentration may have been a factor, since the carbon-filtered water they used in their experiment was relatively soft (26 mg/L $CaCO_3$ vs. 56 mg/L $CaCO_3$ in reconstituted reverse osmosis water), and previous studies have linked scoliosis and hind limb deformities to the rearing of tadpoles in water with calcium <4 mg/l or in pure deionized water (Pollack & Liebig, 1989; Marshall et al., 1980).

We found that supplementing carbon-filtered water with either folic acid or vitamin B complex reduced SLS prevalence, which is consistent with folic acid or other B vitamins mitigating any negative effects of carbon-filtered water on SLS. No mechanism has been proposed to explain the observation that vitamin B supplementation of tadpole rearing water reduced the prevalence of SLS in *Epipedobates* spp., *Dendrobates* ssp., and *Phyllobates* spp. (Wright & Whitaker, 2001). However, each B vitamin (B1, B2, B3, B6, folate, and B12) plays a unique role in bone physiology (Dai & Koh, 2015), in particular folic acid and vitamin B12, which are cofactors for the enzymes that determine homocysteine (an amino acid risk factor for osteoporosis and fractures in humans) levels (Clarke et al., 2014; Dai & Koh, 2015; Herrmann et al., 2007; Mclean & Hannan, 2007; Wildermann & Herrmann 2007). Further focused study to determine the importance of various B vitamins on the bone health of developing amphibians could improve our understanding of SLS.

7.2 | Experiment 2

Increased husbandry intensity decreased the metamorphosis time of *A. geminisae* tadpoles by approximately 1 month. This effect is likely due to the differences in tadpole feeding frequency, which has been shown to decrease tadpole metamorphosis time across multiple studies (Griffiths et al., 1993; Hota & Dash, 1981; Murray, 1990) whereas the water quality parameters have no known clear effect on tadpole metamorphosis time (Egea-Serrano et al., 2012; Marco & Blaustein, 1999). Regardless of the mechanism, this finding demonstrates that small increases in husbandry workload can reduce the amount of time it takes to rear tadpoles to metamorphosis.

Husbandry may have had other effects too; 30% (9 out of 30) of tadpoles in the medium husbandry treatment died before metamorphosis compared with 20% (6 out of 30) of tadpoles in the intensive husbandry treatment. Though not significant, tadpole water in the medium husbandry treatment had elevated levels of nitrite and ammonia, which are known to lower amphibian larvae survival (Egea-Serrano et al., 2012; Huey & Beitinger, 1980; Odum & Zippel, 2011). We note that dendrobatid tadpoles may be special; while general amphibian husbandry guidelines warn against nitrite levels exceeding 0 ppm (Wright & Whitaker, 2001), typical nitrite concentration of bromeliad water containing the dendrobatid *Ranitomeya amazonica* tadpoles is about 10 ppm (Poelman et al., 2013).

Ca^{2+} sequestering time had a significant negative effect on SLS. This suggests that changes in water chemistry associated with the failure and repair of the phosphate reactor had an important effect on SLS. We hypothesize that excess phosphate exposure during the time that the phosphate reactor was broken reduced the amount of dissolved calcium available to developing tadpoles, causing SLS. We base this hypothesis on the physiology of tadpoles during metamorphosis and the relationship between the phosphate: calcium ratio and SLS.

Calcium is vital for bone ossification during amphibian metamorphosis (Baldwin & Bentley, 1980; Oguro et al., 1975; Pilkington & Simkiss, 1966; Stiffler, 1993). Tadpoles acquire environmental calcium across the skin (Guirguis et al., 1997; Stiffler et al., 1997; Stiffler, 1995, 1996; Zerella & Stiffler, 1999) and gills (Baldwin & Bentley, 1980; Stiffler, 1993, 1996), and via dietary calcium through the wall of the small intestine (Robertson 1975, 1976; Stiffler, 1993, 1996). However, during peak metamorphosis, external gills are gone and tadpoles are unable to feed due to the restructuring of the mouth (Gosner, 1960; Pilkington & Simkiss, 1966). Tadpoles store calcium as calcium carbonate in specialized endolymphatic sacs in the inner ear, mobilized during metamorphic climax (Pilkington & Simkiss, 1966). In *Rana temporaria*, the calcium concentration of tadpole rearing water is directly correlated with both the amount of calcium carbonate in the endolymphatic sacs and the degree of skeleton mineralization in metamorphs (Pilkington & Simkiss, 1966). The availability of calcium and the window of time tadpoles have to accumulate calcium should impact the health of bones that develop during metamorphic climax; our Ca^{2+} sequestering time variable may capture the window of time that each tadpole had to sequester calcium before metamorphic climax.

Camperio Ciani et al. (2018) noted that the phosphate:-calcium ratio differed between the water filtration treatments associated with SLS prevalence (1:986 in reconstituted reverse osmosis water, associated with low SLS compared with 1:189 in carbon-filtered water, associated with high SLS). More recently, Lassiter et al. (2020) experimentally demonstrated that lowering the phosphate: calcium ratio in tadpole rearing water significantly reduced the incidence of SLS in *Atelopus varius* tadpoles. We also noted a correlation between changes in the phosphate: calcium ratio of tadpole rearing water (caused by the malfunction of the phosphate reactor) and SLS. The tadpole source water analyzed during Experiment 2 confirms that phosphates decreased from ~0.4 to 0–0.04 ppm PO_4^{3-} following reactor repair, while calcium levels remained relatively constant. This decrease in the phosphate: calcium ratio was accompanied by a drop in the prevalence of SLS. This is consistent with our estimates of the phosphate: calcium ratio before and after phosphate removal from our July 2018 analysis, which showed phosphate levels in the tadpole rearing water remained at about 0.54 ppm PO_4^{3-} without a functioning phosphate reactor, compared with 0.04 ppm PO_4^{3-} with a functioning phosphate reactor, while calcium levels remained between 15 and 20 ppm $CaCO_3$. Our phosphate and calcium data from individual tadpole wastewater did not reflect this pattern, likely due to the cyclic introduction and removal of phosphates through food waste and feces and/or the limited accuracy of the test strips we used for these measurements. Nonetheless, the broken reactor increased the phosphate:calcium ratio of the water used for tadpole rearing, and this was correlated with an increased prevalence of SLS.

An important anecdote is that the same phosphate reactor that failed during our second experiment was installed at the PARCP near the beginning of Experiment 1, where only six cases of SLS appeared across 87 tadpoles over 6 months despite historical levels of 58% SLS in this population (unpublished 2016–2017 PARCP data). The new phosphate reactor may have led to the surprising low levels of SLS in Experiment 1 by decreasing the phosphate:calcium ratio. In Experiment 2, four tadpoles raised in carbon-filtered water under nearly identical care conditions as Experiment 1 developed SLS in succession within the first 4 months of the experiment—the same time period during which the phosphate reactor was broken. We posit the malfunction of this reactor led to the initially high levels of SLS in Experiment 2 by increasing the phosphate:calcium ratio.

While we found that husbandry treatment had no significant effect on the prevalence of SLS in Experiment 2, it did interact with calcium sequestering time, with medium husbandry dampening the effect of calcium sequestering time on SLS. However, tadpoles in the medium husbandry treatment had much longer development times, and so experienced higher and less varied calcium sequestering times overall than tadpoles in the intensive husbandry treatment (medium husbandry treatment mean calcium sequestering time = 48 days,

SD = 18.5, vs. intensive husbandry treatment mean calcium sequestering time = 22.1 days, *SD* = 24.5). While this interpretation implies that calcium sequestering time and husbandry are somewhat confounded in our analyses, the observed pattern is contrary to our expectation, where SLS prevalence was somewhat lower (not higher) in the medium husbandry treatment.

8 | CONCLUSION

In Experiment 1, we found that the filtration method and vitamin supplementation of tadpole rearing water may mediate the prevalence of SLS. Specifically, we found that the use of carbon-filtered water to rear tadpoles aggravates SLS, but that supplementing carbon-filtered water with either folic acid or a vitamin B complex may neutralize any negative effects of carbon filtration. In Experiment 2, we found that less intensive tadpole husbandry increased metamorphosis time by approximately 30 days and we observed an association between elevated phosphate-to-calcium levels in the tadpole rearing water and elevated SLS prevalence. The change in the phosphate:calcium ratio of the tadpole rearing water was unintentional and so we are not presenting a new putative SLS factor. However, in light of multiple instances of correlation between an increased phosphate:calcium ratio in tadpole rearing water and increased prevalence of SLS and the recent experimental demonstration of this phenomena by Lassiter et al. (2020), the body of data currently available points to phosphate-induced calcium deficiency as a candidate hypothesis for the etiology of SLS and provides ex-situ conservation projects with important information to consider when designing husbandry protocols for populations afflicted by SLS.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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